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Analysis of the susceptibility of CD57$^+$ T cells to CD3-mediated apoptosis

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Summary

After stimulation with anti-CD3 antibody *in vitro*, CD57$^+$ T cells showed a greater susceptibility to apoptosis than CD57$^-$ αβT cell receptor (TCR)$^+$ T cells (regular αβ T cells). The apoptotic fraction of CD57$^+$ T cells showed an increased production of active caspase-3. An increase in both Fas expression and Fas-ligand (FasL) production was also observed in CD57$^+$ T cells, whereas the expression of survivin was suppressed in CD57$^+$ T cells compared to that of regular αβ T cells. CD57$^+$ T cells display a biased expansion of a few Vβ T cell fractions in individuals, but such Vβ T cells were not specifically susceptible to CD3-mediated apoptosis. The TCR expression level of CD57$^+$ T cells was much lower than that of regular T cells and anti-TCR antibody stimulation induced a smaller apoptotic proportion of CD57$^+$ T cells than did anti-CD3 antibody. Although the CD3ε expression levels were similar in both T cell subsets, the CD3ζ level of CD57$^+$ T cells was significantly higher than that of regular T cells. These results suggest that several apoptotic and anti-apoptotic molecules are involved in the CD3-induced apoptosis of CD57$^+$ T cells and raise the possibility that the imbalance in expression of the CD3ε and CD3ζ chains may also contribute to the susceptibility of CD57$^+$ T cells to undergo apoptosis.

Keywords: CD57$^+$ T cells, CD3-mediated apoptosis, Fas/FasL, NKT cells, survivin

Introduction

Human T cells with a natural killer cell marker, CD57 [1], are known to increase with ageing and have been suggested to differentiate extrathymically [2–5]. Because CD57$^+$ T cells, most of which are CD8$^+$, have a capacity to produce a larger amount of interferon (IFN)-γ than regular αβ T cells [4], they seem to play an important role in the immunological changes with ageing, and may therefore affect the T helper 1 (Th1)/T helper 2 (Th2) balance.

However, human CD57$^+$ T cells and another type of human natural killer (NK)-type T cell, namely CD56$^+$ T cells, have been shown to kill not only tumours but also vascular endothelial cells when activated with cytokines or bacteria superantigens [6,7] while also producing a large amount of Fas-ligand (FasL) [8]. Furthermore, these human NK-type T cells are also more susceptible to apoptosis after CD3/T cell receptor (TCR) cross-linking than ordinary T cells [4]. Using a mouse model, we also reported recently that natural killer 1·1Ag (NK1·1)$^+$ T cells stimulated with a synthetic ligand, α-galactosylceramide, produce IFN-γ to activate NK cells and CD8$^+$ T cells and kill tumours [9–11], whereas Fas-ligands produced by them cause the severe hepatic injury. Furthermore, these functions of NK1·1$^+$ T cells become enhanced with mouse ageing [10]. However, NK1·1$^+$ T cells rapidly undergo apoptosis after activation, probably not to induce further tissue injuries [9,12], although it has been reported recently that in some animal models spleen and liver NK T cells do not undergo apoptosis after α-galactosylceramide or IL-12 stimulation but instead become phenotypically inactive because of the down-regulation of NK1·1 and internalization of T cell receptors [13–15]. Although NK-type T cells both in humans and mice are important effectors against tumours and infections by inducing the Th1 immune response, they may be autoreactive to eliminate abnormal cells and senescent cells in aged hosts, and they may thus need to rapidly undergo apoptosis or to down-regulate their molecules not to induce further

tissue damage. It is therefore important to elucidate the mechanism of apoptosis of CD57+ T cells to obtain a better understanding of their physiological behaviour and roles in the immunology of elder hosts. It is known that activation of T cells results in the increased expression of apoptosis-inducing molecules including Fas (CD95) and FasL [16–18].

In the present study, we demonstrate the unique features of CD57+ T cells, in view of apoptosis-related molecules regarding such factors as the caspase-3 activity, Fas/FasL expression and survivin expression. We also show an imbalance of CD57+ T cells in the expression between CD3ε and CD3ζ molecules and such an imbalance may be involved in the susceptibility of CD57+ T cells to CD3-induced apoptosis.

Materials and methods

Cell sorting and culture

Heparinized peripheral blood samples were obtained from adult volunteers. Prior to the blood collection, the aim and details of the experiments were explained thoroughly and consent was obtained from all subjects. Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood by Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway). Surface phenotypes of the PBMC were identified by monoclonal antibodies in conjunction with three-colour immunofluorescence tests. For sorting experiments, PBMC were stained with PE-anti-αβ TCR antibody, FITC-anti-CD57 antibody and PC5-anti-CD56 antibody. Next, CD56$^-$ CD57$^+$αβ TCR$^+$ cells (CD57$^+$ T cells) and CD56$^-$CD57$^-$αβ TCR$^+$ cells (regular αβ T cells) were purified with a fluorescence-activated cell sorter (EPICS Elite, Beckman Coulter, Fullerton, CA, USA). The purity of each population was more than 95%. One hundred μl (5 μg/ml) of anti-CD3 antibody (UCHT1) were incubated at 37°C for 4 h in 96-well flat-bottomed plates to immobilize the antibody before starting the culture. The cells of each T cell population (1×10^5 in 100 μl of RPMI 1640 containing 20% human serum) were cultured with immobilized anti-CD3 antibody in a 96-well flat-bottomed plate. The cells were harvested serially and then subjected to the experiments described below. In the case of anti-αβ TCR stimulation, 100 μl (5 μg/ml) of anti-TCR pan αβ (BMA031) was immobilized to the culture plate and used for the experiments. In some cases, lymphocytes were cultured with anti-IFN-γ or 5 ng/ml of interleukin (IL)-15 (Genzyme).

Assay for lymphocyte apoptosis

An assay for reactivity to annexin V in apoptotic cells was performed using commercial reagents (Immunotech, Marseille, France) according to the manufacturer's instructions. After staining the cells with FITC-annexin V and propidium iodide, the cells were applied to a flow cytometer (EPICS XL, Beckman Coulter).

The caspase-3 activity of the lymphocytes was evaluated as the protease activity of caspase-3 by using the PhiPhiLux-G_1D_2 kit (OncoImmunin, Inc., Gaithersburg, MD, USA) after *in vitro* cultivation. PhiPhiLux-G_1D_2 (GDEVDGI fluorogenic heptapeptide), a substrate for the caspase-3, can penetrate into the cell nucleus and is converted to the fluorescent form when it is cleaved by the protease activity of caspase-3. The cells were incubated with PhiPhiLux G1D2 for 1 h at 37°C and then stained with PI. The caspase-3 activity in lymphocytes was analysed by cytometer.

Analysis of CD95 expression

Monoclonal antibody against CD95 (clone UB2, Beckman Coulter) was used to detect the expression level of Fas molecules on the cultured lymphocytes. The Fas expression was evaluated as the mean fluorescence intensity calculated from the flow cytometry results.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of survivin and Fas-ligand (FasL)

Total RNA was isolated from 1×10^6 cells using a GlassMAX® RNA Microisolation Spin Cartridge System (Life Technologies, Inc., Rockville, MD, USA) according to the instruction manual. RNA (0·5 μg) was reverse transcribed with a SuperScript One-Step RT-PCR™ System (Life Technologies, Inc.). The RT reaction was performed at 45°C for 30 min and was then terminated by heating to 94°C for 2 min. PCR consisted of 40 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 1 min, and extension at 72°C for 1 min. The sequence of the oligonucleotide primers were as follows: survivin-forward (5′-AGGACCACCGCATCTCTAC-3′), survivin-reverse (5′-ACTTTCTTCGCAGTTTCCTC-3′), FasL-forward (5′-CACCCCAGTCCACCCCCTGA-3′), FasL-reverse (5′-AGGGGCAGGTTGTTGCAAGA-3′), GAPDH-forward (5′-GTGAAGGTCGGAGTCAACG-3′), and GAPDH-reverse (5′-GGTGAAGACGCCAGTGGACTC-3′). The PCR products were separated on 2% agarose gel and were then transferred to a nylon membrane (Immobilon-S, Millipore Corporation, Bedford, MA, USA) with a semidry electroblotter (Nihon Eido Co. Ltd, Tokyo, Japan). Next, the PCR products were probed with a digoxigenin (DIG)-labelled internal probe (survivin internal probe: 5′DIG-CACTGCCCCACTGAGAAC-3′; FasL internal probe: 5′DIG-CTGGAATGGGAAGACACCT-3′) and visualized using the DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. In the case of GAPDH (used as an internal standard), the agarose gel was stained with ethidium bromide and visualized by UV light.

Analysis of Vβ TCR repertoire of regular αβ T cells and CD57+ T cells

The cells were analysed by three-colour flow cytometry using PE-anti-αβ TCR antibody, PC5-anti-CD56 antibody, FITC-

anti-CD57 antibody and various PE-anti-Vβ TCR antibodies (Vβ1, 2, 5·1, 8, 9, 14, 17 and 22) (Beckman Coulter). Anti-Vβ TCR antibodies that reportedly reacted with relatively larger populations of αβ T cells were selected and used in this study. The percentage of each Vβ T cell population was determined as follows:

% of Vβ T cells in regular αβ T cells = (% $CD56^-CD57^-$Vβ T cells/% $CD56^-CD57^-$αβ T cells) × 100

% of Vβ T cells in $CD57^+$T cells = (% $CD56^-CD57^+$Vβ T cells/% $CD56^-CD57^+$ αβ T cells) × 100

Expression of αβ TCR, CD3ε and CD3ζ molecules

The expression of αβ TCR and CD3ε molecules on the $CD57^-$ (regular αβ) T cells and $CD57^+$T cells was examined by a regular three-colour fluorescence-based surface marker analysis. The expression of intracellular CD3ζ molecules was examined by the techniques as described in the instruction manual. In brief, the PBMC were stained with membrane-specific conjugated antibodies (FITC-anti-CD57 and PC5-anti-αβ TCR) and incubated for 30 min at room temperature in the dark. After washing, the cells were fixed with 0·25% formaldehyde-phosphate-buffered saline (PBS) for 10 min. Then the membrane was then permeabilized by digitonin (100 μg/ml) for 15 min on ice. The intracellular component of ζ molecules in the CD3 complex was stained by PE-antiζ monoclonal antibody (clone 2H2D9, TIA-2, Immunotech) in a saturating concentration. In each case, the stained cells were assessed by a flow cytometric analysis, and then the mean fluorescence intensity of the αβ TCR, CD3ε and CD3ζ molecules was measured.

Statistical analysis

Differences between the two groups (regular αβ T cells and $CD57^+$ T cells) were analysed by Student's *t*-test and were considered to be significant when $P < 0{\cdot}05$.

Table 1. Apoptotic ratio after CD3 stimulation in mixed culture of $CD57^+$ T cells and regular αβ T cells.

Time after CD3 stimulation	Apoptotic ratio (% of annexin V-positive cells)*		
	$CD57^+$ T cells	$CD57^-$αβ T cells**	*P*-value†
Day 1	42·8 ± 3·4	20·4 ± 7·0	<0·05
Day 2	27·3 ± 2·0	10·5 ± 2·5	<0·001

*Unsorted whole PBMC were stimulated with anti-CD3 antibody and apoptotic fraction was analysed by a three-colour flow cytometry. Apoptotic ratio was calculated as annexin V-positive fraction in $CD57^+$αβ TCR^+ ($CD57^+$ T) cells or $CD57^-$αβ TCR^+ ($CD57^-$αβ T) cells. **In this analysis $CD57^-$αβ T cells may include $CD56^+$ T cells, which are also susceptible to CD3 stimulation-induced apoptosis [4]. Nevertheless, $CD57^+$ T cells show much higher apoptotic ratio than $CD57^-$αβ T cells. †*P*-value was analysed by a Student's *t*-test ($n = 3$).

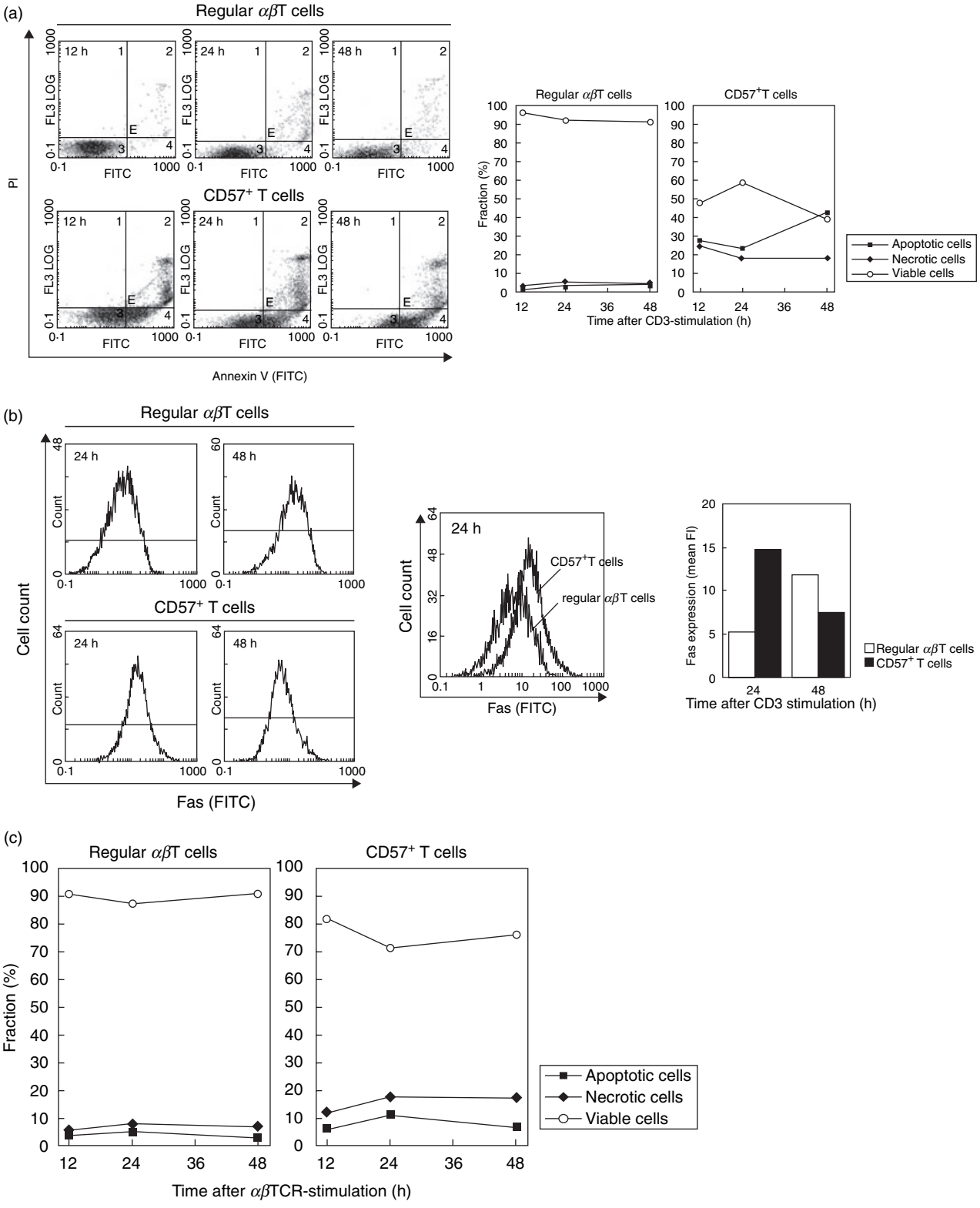
Results

High susceptibility of $CD57^+$ T cells to apoptosis in response to CD3-stimulation

Purified regular αβ T cells and $CD57^+$ T cells were stimulated with anti-CD3 antibody and the susceptibility to apoptosis was compared by a flow cytometric analysis using PI and FITC-annexin V staining (Fig. 1a, left). αβ T cells maintained a high viability (>90%) during the observation period and the frequency of apoptotic cells was very small. In contrast, a remarkable increase in annexin V-positive (apoptotic) or both annexin V- and PI-positive (post-apoptotic necrosis) fractions was observed in $CD57^+$ T cells from 12 h after CD3-stimulation. The apoptotic fraction reached more than 40% of the cultured $CD57^+$ T cells at 48 h (Fig. 1a, right). This suggests that apoptotic cell death and post-apoptotic necrosis were actively induced in $CD57^+$ T cells after stimulation with anti-CD3 antibody.

To confirm whether $CD57^+$ T cells are really more prone to undergo apoptosis than regular αβ T cells, the apoptotic ratio of $CD57^+$ T cells in co-cultures containing regular αβ T cells was measured (Table 1). The apoptotic ratio of CD3-

Fig. 1. Apoptosis and apoptosis-related molecules of $CD57^+$ T cells after stimulation with anti-CD3 antibody or anti-αβ TCR antibody. (a) Time-course of CD3-stimulated apoptosis in regular αβ T cells and $CD57^+$ T cells. Representative results are shown from repeated experiments with similar results. Left: each T cell population was stimulated with anti-CD3 antibody for 12, 24 and 48 h and stained with propidium iodide (PI) and FITC-annexin V and was then analysed by flow cytometry. Right: the percentages of the apoptotic (annexin V-positive and PI-negative) cells, necrotic (PI-positive) cells and viable (both annexin V and PI-negative) cells were calculated from the results of the flow cytometric analyses and displayed as a function of the time after CD3-stimulation. (b) The expression of cell-surface Fas molecules in regular αβ T cells and $CD57^+$ T cells after CD3-stimulation. Left: flow cytometry results for expression of Fas in each T cell population at 24 and 48 h after stimulation with anti-CD3 antibody. Middle: a histogram overlay of the results of regular αβ T cells and $CD57^+$ T cells at 24 h. Note the increased expression of Fas molecules on the surface of $CD57^+$ T cells. Right: the Fas level was expressed as the mean fluorescence intensity (FI) and displayed on the graph. (c) Time-course of anti-αβ TCR-stimulated apoptosis in regular αβ T cells and $CD57^+$ T cells. Representative results are shown from repeated experiments with similar results. Each T cell population was stimulated with anti-αβ TCR antibody for 12, 24 and 48 h and stained with PI and FITC-annexin V and then was analysed by flow cytometry. The percentages of the apoptotic cells, necrotic cells and viable cells were calculated and displayed as a function of the time after αβ TCR-stimulation.


(a)
Regular αβT cells
12 h
24 h
48 h
FL3 LOG
FITC
PI
CD57+ T cells
Annexin V (FITC)
Regular αβT cells
CD57+T cells
Fraction (%)
Time after CD3-stimulation (h)
Apoptotic cells
Necrotic cells
Viable cells
(b)
Regular αβT cells
Count
CD57+ T cells
Cell count
Fas (FITC)
24 h
CD57+T cells
regular αβT cells
Fas expression (mean FI)
Regular αβT cells
CD57+ T cells
Time after CD3 stimulation (h)
(c)
Regular αβT cells
CD57+ T cells
Fraction (%)
Apoptotic cells
Necrotic cells
Viable cells
Time after αβTCR-stimulation (h)

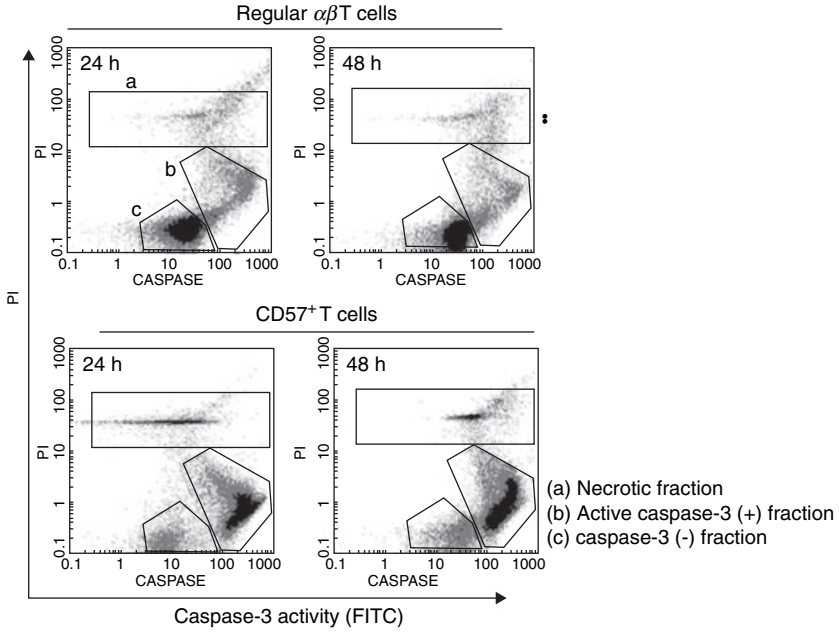


Fig. 2. The expression of active caspase-3 in regular αβ T cells and CD57$^+$ T cells after CD3-stimulation. 24 and 48 h after stimulation of each T-cell population with anti-CD3 antibody, intracellular activation of caspase-3 was examined by using a PhiPhiLux-G_1D_2 substrate.

stimulated CD57$^+$ T cells showed a much higher value than CD57$^-$αβ T cells ($P < 0{\cdot}05$ at day 1 and $P < 0{\cdot}001$ at day 2). This means that CD57$^+$ T cells are highly apoptotic in their nature even in the presence of other supporting cells such as regular αβ T cells.

Surface expression of the Fas molecules after CD3-stimulation

The surface expression of the Fas molecules is one of the most important factors to assess the susceptibility of the cells to apoptosis because this molecule is proved to be involved directly in the activation of caspase-3, a key enzyme in the execution of DNA fragmentation. Therefore, the surface expression of the Fas molecules in the purified regular αβ T cells and CD57$^+$ T cells after CD3-stimulation was observed by a flow cytometric analysis (Fig. 1b). The expression level of the Fas molecules in regular αβ T cells remained at a low level 24 h after CD3-stimulation, and it then increased at 48 h. In contrast, Fas expression on the surface of CD57$^+$ T cells was remarkably up-regulated at 24 h, and thereafter it decreased at 48 h (Fig. 1b, left). An overlay histogram analysis (Fig. 1b, middle) revealed that the fluorescence intensity of the Fas molecules in CD57$^+$ T cells at 24 h was significantly higher than that in regular αβ T cells (14·8 *versus* 5·2) (Fig. 1b, right).

Susceptibility of CD57$^+$ T cells to the apoptotic cell death after αβ TCR-stimulation

Purified regular αβ T cells and CD57$^+$ T cells were stimulated with anti-αβ TCR antibody and the susceptibility to apoptosis was compared by a flow cytometric analysis using PI and FITC-annexin V staining (Fig. 1c). Similar to the case of anti-CD3 stimulation, αβ T cells maintained a high viability (about 90%) during the observation period and the frequency of apoptotic cells was very small. In the case of CD57$^+$ T cells, unlike anti-CD3 stimulation, only 6–11% of the cells were apoptotic, and the ratio of the necrotic fraction remained below 18% throughout the observation period. This strongly suggests that CD57$^+$ T cells are relatively resistant to apoptotic cell death after stimulation with anti-αβ TCR antibody in comparison to anti-CD3-stimulation.

Up-regulation of caspase-3 activity in the apoptotic CD57$^+$ T cells

To confirm the intracellular activation of the apoptosis-related proteases, a caspase-3 activity was observed using a fluorogenic substrate PhiPhiLux-G_1D_2 (Fig. 2). A remarkable increase in the active caspase-3-positive fraction was detected exclusively in CD57$^+$ T cells. This result indicates that the apoptosis-related signalling pathway is actively up-regulated in the annexin V-positive CD57$^+$ T cells after anti-CD3 stimulation.

mRNA expression of survivin and FasL after CD3-stimulation

An increased Fas expression in CD57$^+$ T cells after stimulation with anti-CD3 antibody indicates that these cells become sensitive to FasL and undergo activation-induced cell death (AICD). In most cases, the Fas-FasL signalling in these cells is considered to be carried out in an autocrine

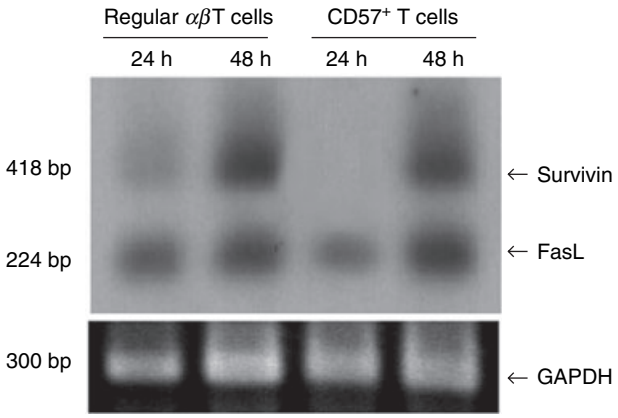


Fig. 3. Expression of survivin and FasL in regular αβ T cells and CD57+ T cells after CD3-stimulation. mRNA was harvested at 24 and 48 h, and RT-PCR for survivin and FasL was performed. GAPDH was used as an internal standard.

manner. To confirm anti-apoptotic and pro-apoptotic events in the CD3-stimulated lymphocytes, mRNA expression of survivin and FasL was investigated in the purified regular αβ T cells and CD57+ T cells (Fig. 3). The expression of survivin mRNA in regular αβ T cells was detectable from 24 h and it was up-regulated markedly at 48 h. In contrast, the expression of survivin mRNA in CD57+ T cells was undetectable at 24 h although it became detectable at 48 h. In case of FasL mRNA expression, remarkable RT-PCR bands were detected in both regular αβ T cells and CD57+ T cells from 24 h. The imbalance between anti-apoptotic molecules (survivin) and pro-apoptotic molecules (FasL) may explain the high susceptibility to apoptosis in CD57+ T cells. According to the annexin V analysis, the apoptotic ratio of CD57+ T cells increased dramatically at 48 h (Fig. 1a) and this was also supported by the results of the caspase-3 activity (Fig. 2). However, there was a discrepancy between the time-course of survivin expression and these results. One reason that the survivin mRNA of CD57+ T cells is detectable at 48 h is that most mRNA seems to be derived from viable (non-apoptotic) cells while less mRNA is derived from apoptotic cells at this time-point.

Vβ repertoires of CD57+ T cells after CD3-stimulation

A Vβ T cell repertoire analysis of the peripheral blood lymphocytes revealed that the biased expansion of a few Vβ T cells occurred in CD57+ T cells but not in regular αβ T cells (Fig. 4a). To observe the susceptibility of these expanded Vβ T cells to apoptosis, purified regular αβ T cells and CD57+ T cells were stimulated with anti-CD3 antibody, and a Vβ T cell repertoire analysis was performed in both apoptotic and proliferative fractions (Fig. 4b). In regular αβ T cells, any Vβ-specific occurrence of apoptotic cell death was not observed in cases A and D. Regarding the clonality of CD57+ T cells, Vβ2 and Vβ8 were expanded selectively in the periphery in cases A and D, respectively. After stimulation with anti-CD3 antibody, neither a specific decrease of these Vβ T cells in the proliferative fraction nor a specific increase of Vβ T cells in the apoptotic fraction was observed. This suggests that highly apoptosis-susceptible cells in CD57+ T cells are not restricted to a few Vβ T cell fractions expanded.

Expression of αβ TCR, CD3ε and CD3ζ in regular αβ T cells and CD57+ T cells

Since regular αβ T cells and CD57+ T cells showed a different susceptibility to apoptotic cell death after CD3-stimulation, and also CD57+ T cells showed a different susceptibility to apoptosis between CD3-stimulation and αβ TCR-stimulation, the expression of αβ TCR and CD3 molecules, which are involved in the transduction of signals after the TCR engages its ligand, was compared between these two lymphocyte groups (Table 2). No remarkable difference in the expression level of CD3ε chain, an extracellular component of the CD3 molecule, was recognized between the two groups. On the contrary, there was a significant increase in the expression of the CD3ζ chain, an intracellular signal-transducing component of the CD3 molecule, in CD57+ T cells ($P < 0{\cdot}05$). However, the expression level of αβ TCR in CD57+ T cells was far below that observed in regular αβ T cells ($P < 0{\cdot}0001$). Consistent with the low TCR level of CD57+ T cells, the anti-TCR antibody stimulated proliferation of CD57+ T cells was significantly lower than that induced by anti-CD3 stimulation, while the difference was not so evident in regular CD57− T cells (not shown).

Discussion

CD57+ T cells constitute approximately 20% of normal human CD8+ T cells. This population increases dramatically in patients after organ transplantation, with rheumatoid arthritis, AIDS [19–23] and ageing [4]. Although their functions have yet to be elucidated fully, CD57+ T cells should participate in the host defence mechanisms including anti-tumour and anti-infectious activities because they are potent antitumour effectors and IFN-γ producers and thereby PBMC from elderly people produce a larger amount of IFN-γ after CD3-stimulation than do PBMC from younger people [4,8].

However, regardless of their IFN-γ producing capacity, CD57+ T cells displayed a poor proliferative response and high susceptibility to apoptotic cell death when stimulated with anti-CD3 antibody (Figs 1a, 2). In contrast, anti-CD3 antibody was strongly mitogenic for CD57− regular αβ T cells and they also maintained a high viability. Several past studies have emphasized that the poor ability of CD57+ T cells to proliferate in response to mitogenic lectins and to stimulation by CD3 antibodies was ascribed to a lack of IL-2 secretion [24,25]. In other reports, the CD57 expression on

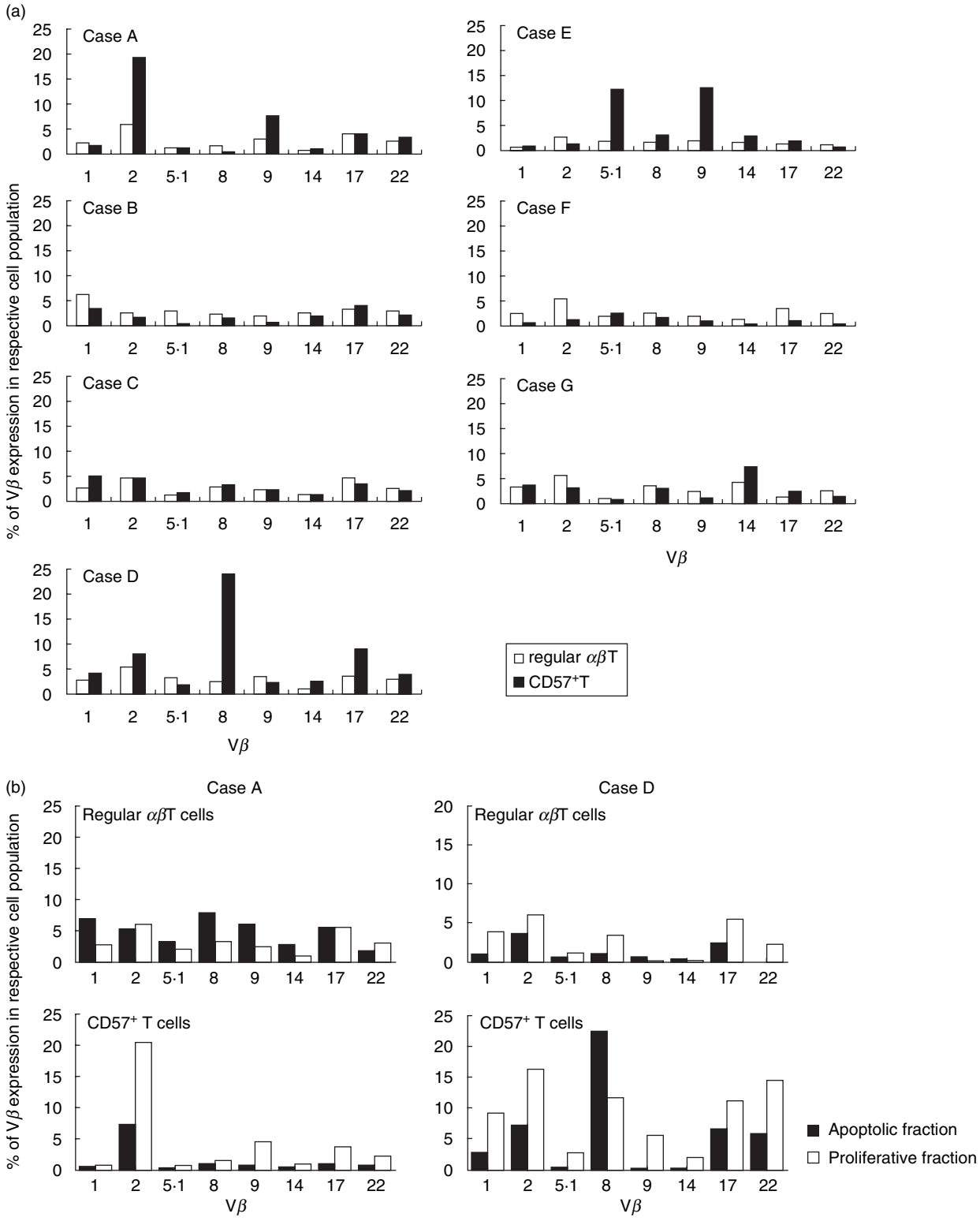


Fig. 4. Vβ repertoires of CD3-stimulated CD57$^+$ T cells. (a) T cell receptor β repertoire of regular αβ T cells and CD57$^+$ T cells. PBMC from seven individual healthy volunteers were stained as described in Materials and Methods, and the percentage of Vβ T cells in respective cell populations were compared. Note that an oligoclonal expansion of certain Vβ T cells was observed only in CD57$^+$ T cells (cases A, D and E). (b) A Vβ repertoire analysis of regular αβ T cells and CD57$^+$ T cells after CD3-stimulation. Each T cell population was stimulated with anti-CD3 antibody for 48 h, and the percentage of Vβ T cells in apoptotic and proliferative cell populations was compared. In the cell scatter analysis apoptotic cells were recognized as the fraction of low forward-scatter (FSC) and high side-scatter (SSC), whereas proliferative population was recognized as that of high FSC and intermediate SSC. Representative results (case A and case D) are shown in this figure.

$CD8^+$ T cells, $CD4^+$ T cells and NK cells is reported to be a general marker of proliferative inability [26], while the $CD57^+$ subset is considered mainly to represent recently activated effector T cells [27]. These findings suggest that $CD57^+$ T cells are terminally differentiated and less proliferative. However, another study suggested that $CD57^+$ T cells can demonstrate full autocrine proliferation if multiple accessory signals are brought to the cells [28]. Therefore, it has not yet been elucidated clearly regarding whether $CD57^+$ T cells are really more susceptible to undergo apoptosis than regular αβ T cells or they are simply more easily activated and fully differentiated after *in vitro* stimulation. In our previous studies, however, after stimulation with IL-2, IL-12 and IL-15, $CD57^+$ T cells produce more IFN-γ than normal $CD8^+$ T cells [4,8]. This suggests that purified $CD57^+$ T cells do express commonγ-chain associated receptors and are responsive to their related cytokines. However, the addition of IL-15 in the culture system cannot prevent CD3-mediated cell death of $CD57^+$ T cells (data not shown). Therefore, $CD57^+$ T cells seem to respond to exogenous cytokines through γ-chain associated receptors but they are prone to undergo apoptosis. We performed additional experiments in which unsorted PBMC were stimulated by anti-CD3 antibody. $CD57^+$ T cells show a very high apoptotic (annexin V positive) rate even under the support of regular αβ T cells that are far less susceptible to apoptosis (Table 1). Taken together, we conclude that $CD57^+$ T cells are naturally a highly apoptosis-susceptible subset.

To determine what factors are involved in the susceptibility to apoptosis of $CD57^+$ T cells after CD3-stimulation, we investigated the expression of Fas molecules. Some reports have suggested alterations in activation-induced apoptosis of lymphocytes in ageing [29,30] and the involvement of the Fas-mediated mechanism in increased apoptosis of T cell subsets in aged humans has also been suggested [31]. Our results revealed that the Fas expression on the surface of $CD57^+$ T cells was up-regulated remarkably within 24 h and that Fas level remained low in regular αβ T cells (Fig. 1b). Therefore, the involvement of the Fas-mediated mechanism was also suggested strongly in our experiments. We next observed the difference in the expression of FasL between regular αβ T cells and $CD57^+$ T cells, which is expressed predominantly in activated T cells [32–34]. However, FasL mRNA was readily detectable and we could not find any difference in the FasL mRNA levels between the two groups (Fig. 3). This was also confirmed by measuring FasL protein levels in the culture medium (data not shown). This is in contrast to the case of $CD57^+$ T cells stimulated with a combination of IL-2, IL-12 and IL-15 [8], in which $CD57^+$ T cells produce a larger amount of FasL than regular T cells. Although the reason of this discrepancy is unclear at present, Th1 cytokines may induce more strongly the autoreactivity of $CD57^+$ T cells than anti-CD3 antibody.

We next compared the anti-apoptotic activity between regular αβ T cells and $CD57^+$ T cells. Survivin is a recently recognized member of the inhibitor of apoptosis protein (IAP) family [35]. Survivin binds with the terminal effector caspases, namely caspase-3 and caspase-7, and inhibits their protease activity. Survivin can be detected in the majority of lymphocyte lines [35] and it effectively prevents apoptosis induced by Fas signals [36]. Although the expression of survivin mRNA was clearly detected at 48 h, its mRNA level in $CD57^+$ T cells was almost nil at 24 h (Fig. 3). Therefore, soon after stimulation with anti-CD3 antibody, $CD57^+$ T cells are considered to have very weak anti-apoptotic ability.

To avoid any possible harmful effects by continuously activated cells, activated NK-type T cells such as $CD57^+$ T cells may be prone to die rapidly after inducing a Th1-type immune response in the hosts. In fact, $CD57^+$ T cells as well as $CD56^+$ T cells activated by a bacterial superantigen or Th1 cytokines showed cytotoxicities against vascular endothelial cells [6,7]. In addition, $CD57^+$ T cells are main lymphocyte populations that cause large granular lymphocyte leukaemia, in which $CD57^+$ T cells express high levels of Fas/FasL but are resistant to Fas-mediated apoptosis and thereby rheumatoid arthritis-like autoimmune disease may occur frequently in patients with $CD57^+$ T cell leukaemia [37,38]. We suggested previously that human $CD57^+$ T cells are a functional counterpart of mouse $CD8^+CD122^+$ T cells with intermediate TCR [39–41] because of their CD3-induced IFN-γ production capacity and antitumour cytotoxicity and are more susceptible to CD3-induced apoptosis than regular $CD8^+CD122^-$ T cells (our unpublished observation). Furthermore, our previous findings suggest that T cells accumulated in the lymphadenopathy of Fas-mutated *lpr/lpr* mice with the systemic lupus-like disease [42] may be a counterpart of $CD8^+CD122^+$ T cells in normal mice [43,44], suggesting both human $CD57^+$ T cells and mouse $CD8^+CD122^+$ T cells may cause autoimmune diseases under certain conditions. An augmented Fas expression and reduced survivin expression of $CD57^+$ T cells may thus be an important mechanism for their susceptibility to AICD and to regulate both their autoreactivity and tissue damage.

In some diseases, e.g. rheumatoid arthritis and AIDS, $CD57^+$ T cells are reported to increase in the inflammatory sites as well as peripheral blood [21–23]. $CD57^+$ T cells are thought to be autoreactive and they may have a hazardous effect on the hosts who are suffering from autoimmune disorders. The dysregulation of apoptosis in $CD57^+$ T cells might contribute to the pathogenesis or inflammatory process of these diseases. Recently, some reports revealed that the expression of the ζ chain in T or NK cells is reduced in patients with rheumatoid arthritis [45] or malignant diseases [46–48], thus suggesting the importance of the expression level of this molecule under normal conditions. It remains unclear as to whether a decreased level of the ζ chain is involved in the susceptibility of apoptosis in T or NK cells in these diseases, but we think the dysregulation of the ζ chain-associated signals may contribute to the cell survival of $CD57^+$ T cells.

As we have reported recently [8,49], a biased expansion of a few Vβ T cells in CD57$^+$ T cells was found in individuals (Fig. 4a). The expansion may be the result of their activation by a limited set of antigens because expanded Vβ T cells in CD57$^+$ T cells are composed of a few T cell clones [8]. We therefore examined whether these oligoclonally expanded Vβ T cell fractions are susceptible to apoptosis. However, we did not find any correlation between the Vβ T cell repertoire of the CD57$^+$ T cells and susceptibility to AICD (Fig. 4b). The high susceptibility of CD57$^+$ T cells to the AICD is thus considered to be a common feature of the CD57$^+$ T cells themselves and it is not due to the nature of a certain Vβ CD57$^+$ T cell fraction. CD57$^+$ T cells are oligoclonal and increase as age increases [4,5,50]. This suggests that they are resistant to cell death and expand oligoclonally in an actual *in vivo* situation. However, *in vitro* stimulation with anti-CD3 antibody was found to lead CD57$^+$ T cells to undergo dramatic apoptosis, thus suggesting that unphysiological and very strong stimulation may also transduce strong apoptotic signals in an *in vivo* situation. Accordingly, this apoptosis system in CD57$^+$ T cells seems to play an important role in avoiding self-injury.

To investigate further the difference in the susceptibility to apoptosis between regular αβ T cells and CD57$^+$ T cells, receptor analyses were performed. The cell surface expression of the CD3 molecules (CD3ε expression) was almost the same between these two subsets, whereas the expression of the intracellular component of the CD3 molecules, namely CD3ζ, was significantly higher in CD57$^+$ T cells than in regular αβ T cells (Table 2). The ζ chain is involved in the transduction of signals after the T cell receptor (TCR) engages its ligand through the activation of the motif in the cytoplasmic region of this molecule [51]. The ζ chain is also involved in the regulation of the assembly and intracellular transport of the TCR–CD3 complex [52]. Therefore, the expression level of ζ may modulate the function (e.g. activation and maturation) of ζ-expressing lymphocytes. The ζ chain has been been shown recently to decrease in T cells from cancer patients [53,54] and this appears to be greatly attributable to the immunosuppression of patients [55]. Because CD57$^+$ T cells are considered to be potent IFN-γ producers and anti-tumour effectors in the elder hosts, a high expression of the ζ chain may thus facilitate the functional role of CD57$^+$ T cells. In contrast to the high expression of the ζ chain, as we reported recently [8], the expression level of αβ TCR was low in CD57$^+$ T cells in comparison to regular αβ T cells (Table 2), which may reflect the result that CD57$^+$ T cells showed a very small number of apoptotic cells after stimulation with anti-αβ TCR antibody (Fig. 1c).

Table 2. Expression of αβ TCR, CD3ε and CD3ζ in regular αβ T cells and CD57$^+$ T cells.

	Regular αβ T cells*	CD57$^+$ T cells*	*P*-value
αβ TCR	494·6 ± 47·9	272·9 ± 41·4	<0·0001
CD3ε	360·2 ± 36·8	329·5 ± 51·7	n.s.**
CD3ζ	373·9 ± 87·1	446·5 ± 55·2	<0·05

*Expression level of αβ TCR, CD3ε and CD3ζ was observed by a flow cytometric analysis and displayed as the mean fluorescence intensity ± s.d. ($n = 6$); **n.s. = not significant.

There are two candidate molecules on NK T cells that might connect with the ζ chain except TCR. One is CD16 (Fcγ receptor type III), which is more popular in NK cells. CD16 is involved in the cytolytic activity of NK cells and plays an important role in CD2 signal transduction through the ζ chain [56], which contributes to both the adhesion and signal transduction functions in T cells. The other is CD43, which is a cell surface sialoglycoprotein implicated in both haematopoietic cell adhesion and activation. The ζ chain has been proven to function as a scaffold molecule in the CD43 signalling pathway and it activates T lymphocytes as well as NK cells [57]. The distribution of the ζ chains among these receptors inside the CD57$^+$ T cells has not yet been analysed clearly. However, we consider that either an imbalance of the ε chain and ζ chain may affect the CD3 signal transduction or ζ chain-associated signal transduction pathways that do not exist in regular αβ T cells may cross-talk with the CD3 pathway and thereby stimulate the apoptotic pathway in CD57$^+$ T cells.

Taken together, CD3-stimulated CD57$^+$ T cells showed increased induction of pro-apoptotic molecules and a decreased expression of anti-apoptotic molecules presumably to limit their autoreactivity. Imbalanced expression levels of the CD3ζ chain and CD3ε chain and the signal transduction mechanisms via their unique CD3 molecules may also be involved in the susceptibility to apoptosis of CD57$^+$ T cells after CD3-stimulation.

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